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TMZ Resistance in Glioblastoma: Role of P-Glycoprotein

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ABSTRACT

Temozolomide is the most common antineoplastic agent used for glioblastoma therapy. Some patients can develop early resistance to this compound. Overcoming chemoresistance could be an important challenge to improve the prognosis and increase the survival of these patients. The action of some efflux transporters localized on the blood-brain barrier seems to be the main mechanism involved in the resistance. An intriguing member of this resistance mechanism is the P-glycoprotein, an ABC transporter.

In this review, we focus on discussing the role of P-glycoprotein in temozolomide resistance of glioblastoma multiforme. We summarize the current literature on structure, localization and activity of this protein, highlighting its role on the distribution of different drugs used for the treatment of brain tumors and other cancers.

Keywords: P-gp, Temozolomide, Chemoresistance, Glioblastoma, Blood-brain barrier, Multidrug resistance

Abbreviations: P-gp/ABCB1/MDR1: Permeability glycoprotein; GBM: Glioblastoma Multiforme; TMZ: Temozolomide; BBB: Blood-brain Barrier; CNS: Central Nervous System; ABC Transporter: ATP-binding Cassette Transporter; MDR: Multidrug Resistance; BCRP/ABCG2: Breast Cancer Resistance Protein; MRPs: Multidrug Resistance-Associated Proteins; OS: Overall Survival; MGMT: O6-methlyguanine-DNA-methyltransferase; MMR: Mismatch Repair; TMDs: Transmembrane Domains; THMs: Transmembrane α-Helices; NBDs: Nucleotide Binding Domains; CPT-11: Irinotecan; TKIs: Tyrosine kinase Inhibitors; EGFR: Epidermal Growth Factor Receptor; ECF: Extracellular Fluid; siRNA: Small Interfering RNA; RNAi: RNA Interference; lncRNAs: Long Non-Coding RNAs; NPs: Nanoparticles; EGF: Epidermal Growth Factor

INTRODUCTION

GBM is the most common and aggressive primary malignant brain tumor [1,2] with an incidence of 3 cases per 100,000 individuals each year and a median OS less than one year [1,3].

Current standard of care for GBM consists of surgical resection, radiation therapy and chemotherapy. TMZ represents the frontline chemotherapy treatment for GBM [4-6], TMZ together with surgical resection and radiotherapy has improved the prognosis for GBM patients [1,7-10]; however, despite improvements in therapeutic treatment, quality of life and prognosis remain very poor. Moreover the management of GBM patients is complicated by the presence of drug resistance mechanisms that are a common cause for therapeutic failure of several drugs, including TMZ.

TMZ is an oral alkylating chemotherapeutic compound that is able to cross the BBB and acts generating O6-methylguanine adducts which introduce mis-pairs with thymine; it is not possible to repair these adducts which thus create DNA damage resulting in cell cycle arrest, cell death and senescence [4,11-13].

An understanding of the molecular processes associated to resistance is critical to find and develop mechanisms to sensitize GBM cells to TMZ. Several studies try to explain

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TMZ resistance; MGMT and the MMR system appear to be involved in the failure of TMZ treatment [4,13-17].

A possible candidate responsible of resistance to antineoplastic agents, including TMZ, in GBM patients is the P-gp which belongs to the ABC transporter family [18].

P-gp (*ABCB1* or *MDR1*) is an ATP-driven efflux pump which utilizes ATP hydrolysis to transport various substrates across the plasma membrane of several tissues [4]; protein expression has been reported not only in healthy tissues but also in many tumors, including brain tumors [18-23]. Concerning cancer, P-gp is a potent efflux-pump, which through its mechanism of action, is involved in the expulsion of several drugs out of the tumor cells: this mechanism provides an explanation for the resistance of tumor cells to multiple antineoplastic agents known as MDR [4,18,24].

The present review is focused on the role played by P-gp in TMZ resistance of GBM analyzing the molecular and biological mechanisms through which this efflux pump could represent a limiting factor for success of TMZ treatment.

Structure and Function of P-gp

BBB is the greatest challenge in the treatment of CNS tumors, representing the primary obstacle to drug delivery into CNS. It is a dynamic interface that separates the brain from the blood ensuring CNS homeostasis and protecting the brain from potentially harmful substances [18,25,26].

BBB consists of capillary endothelial cells not fenestrated, joined together by tight junctions limiting the passage of solutes [18,27-29]. Moreover brain endothelial cells are characterized by the presence of specific transport systems that regulate the entry of compounds [30]. The major transport system is represented by the ABC transporter family. These efflux transporters are responsible of MDR phenotype, binding and hydrolyzing ATP; BBB is therefore strongly protective for the CNS, but at the same time becomes a limiting factor to treatment of CNS diseases regulating the entry of drugs into the brain.

ABC transporter family is an evolutionarily conserved family of proteins suggesting its paramount role in survival of the species [19,21,31-33]; so far, 49 ABC transporters have been identified and classified in different human tissues [33-36]. The prominent members of this family are P-gp, BCRP (or ABCG2) and MRPs.

P-gp was the first of these transporters to be identified and analyzed [37,38]; it is currently the best known efflux-pump in humans, probably for its significant role in cancer cells chemoresistance. P-gp is coded by the multiple drug resistance *MDR1* gene localized on chromosome 7, it is a single 170 kDa polypeptide [33]. The protein is made of two TMDs, each consisting of six highly hydrophobic TMHs, and two NBDs involved in the binding and hydrolysis of ATP [33,39-41].

P-gp is expressed preferentially in organs with excretory role and in tissues with barrier function, particularly in the apical membrane of epithelial cells including liver, kidney, intestine and apical membrane of endothelial cells of the capillaries of the brain [33]: protein localization suggests a role in the defense of susceptible organs, such as the brain, from toxic compounds and in the secretion of metabolites or xenobiotics [33,42]. In addition P-gp expression was also found in pancreas, adrenal gland, placenta, testis and on the surface of hematopoietic cells [33,43].

P-gp is a plasma membrane protein able to interact with several compounds including chemotherapeutic drugs, immunosuppressive agents, calcium channel blockers and natural products, among many others, pumping them out of the cells [18,33,39,41,44-46]. P-gp substrates differ in size, structure and function, even if most of them are weakly amphipathic and relatively hydrophobic [33,41]. This broad substrate specificity is in agree with P-gp role as efflux pump involved in removing substrates from the inner to the outer side of cell plasma membrane or directly into the extracellular space, preventing the accumulation inside tissues of a variety of compounds, such as drugs, xenobiotics, toxins and metabolites [19,47].

P-gp Influences CNS Distribution of Chemical Agents

Several mechanisms seem to be involved in the poor response of brain tumors to chemotherapy, drugs delivery to the CNS continues to be a clinical challenge.

Some studies have shown the role of P-gp and other ABC transporters, especially BCRP, in preventing therapeutic agents penetration into the brain, including conventional antitumor compounds such as vinca alkaloids, anthracyclines and taxanes [19,48-52]; indeed the expression of these transporters is associated with inherent or acquired MDR [53]. Moreover, recent literature suggests that P-gp and BCRP work together and cooperate at the BBB with a synergistic effect, reducing significantly brain penetration of drugs and consequently their effectiveness [18,54-58].

CPT-11 is an antineoplastic agent which acts inhibiting DNA topoisomerase I, a nuclear enzyme involved in DNA replication, repair and transcription [59, 60]. This compound is strongly used for colorectal cancer treatment and shows an interesting activity against other type of tumors, including GBM [59, 61]. *In vivo* and *in vitro* studies suggested that CPT-11 is able to cross the BBB, but its activity is strongly limited by P-gp action that reduces brain penetration not only of CPT-11, but also of its active metabolite [59].

de Vries et al. demonstrated that BCRP and P-gp work together to reduce plasma exposure and brain penetration of topotecan in a knockout mice model [57]. Topotecan, inhibitor of topoisomerase I, is a derivative of camptothecin;

its efficacy is confirmed in the treatment of ovarian, lung, and cervical cancer and seems to have a moderate effect also in adults with primary malignant glioma [57,62].

Lapatinib, antineoplastic agent, is a member of the 4-anilinoquinazoline class of TKIs; different studies on lapatinib and other TKIs reported the involvement of the efflux transporters of the BBB, among which P-gp, as responsible of low brain concentration of these compounds [63-66]. Regarding TKIs, in another study Agarwal et al. suggested the involvement of P-gp and BCRP on the distribution of an EGFR inhibitor, gefitinib, to the CNS [54]. *In vivo* and *in vitro* experiments showed that both transporters are able to reduce the intracellular accumulation of gefitinib favoring its outflow. As confirmation of these results, brain distribution of gefitinib improved and increased when it was co-administered with a dual P-gp and BCRP inhibitor, suggesting a combined therapy of gefitinib and this type of inhibitors as a novel opportunity for cancer treatment [54].

Another inhibitor of EGFR, erlotinib, is a known substrate of P-gp which prevents its brain penetration; unfortunately erlotinib does not seem to be successful in different clinical trials for GBM [67].

In vivo preclinical studies showed that P-gp reduces the brain penetration of alkaloid compounds too and especially of vinblastine, an antineoplastic agent able to bind to tubulin and inhibit microtubule formation, resulting in disruption of mitotic spindle assembly and arrest of the cell cycle [68]. In particular, the authors compared the pharmacokinetics of vinblastine in P-gp knockout and wild type mice. They observed an increase of drug accumulation in tissues, especially in the brain, and a reduction in drug excretion in P-gp knockout mice, suggesting consequently an involvement of P-gp in the efficacy of vinblastine.

Finally by *in vivo* and *in vitro* studies, P-gp appeared to interfere with novel anticancer molecules too, such as vemurafenib, a BRAF inhibitor approved for the treatment of patients with metastatic melanoma; active efflux of vemurafenib by P-gp and BCRP strongly reduces its brain distribution [69]. These observations could be very useful for the assessment of vemurafenib in the treatment of brain metastasis.

P-gp and Anticancer Strategies

MDR phenomenon in cancer cells is associated to their resistance to a wide range of anticancer compounds, even if structurally and functionally different [33,70,71]. Intrinsic or acquired resistance could be associated to several mechanisms and biological processes such as action of efflux systems, including P-gp and other ABC transporters, enhanced DNA repair, alteration in apoptosis and metabolic modifications [72,73].

Several research studies are conducted with the purpose to find strategies to sensitize cancer cells to chemotherapeutic agents overcoming drug resistance, in particular P-gp inhibitors/modulators have been developed representing a significant opportunity in clinical setting for P-gp-mediated drug resistance.

Different P-gp inhibitors have been identified and some of these compounds may be efficient in cancer treatment in association with other antineoplastic drugs, such as vincristine and daunorubicin [31, 33]. The P-gp inhibitors are classified in different groups depending on potency, selectivity and drug-drug interaction potential; currently it is possible to distinguish four generations of P-gp inhibitors.

About first-generation inhibitors, many compounds belong to this group among which calcium channel blockers, immunosuppressants, anti-hypertensives, antiarrhythmics and antiestrogens [33]. In 1981, Tsuruo et al. using leukemia cells observed that verapamil, a calcium channel blocker, could reverse drug resistance [74]. Verapamil and cyclosporine A are two important examples of early discovery of P-gp inhibitors/modulators, both are P-gp substrates and act competing with other P-gp substrates for efflux by a mechanism of competitive inhibition [33,45]. Unfortunately, the combination of first-generation MDR inhibitors with anticancer drugs leads to toxic side effects, especially serious cardiovascular toxicity; this aspect makes their use clinically difficult, therefore they were replaced by second generation inhibitors [33,45].

Second generation inhibitors were developed in order to increase inhibitory effects and reduce toxicity at the same time. These compounds are analogues of the first generation inhibitors and they were synthesized by structurally modifications of first generation inhibitors. Valspodar is the best known second generation inhibitor, it has been evaluated in association with anticancer drugs in several clinical trials [33,75-90]. Other examples of second-generation inhibitors are non-immunosuppressive analogues of cyclosporin A, R-enantiomer of verapamil and dexverapamil [33]. Despite second-generation P-gp inhibitors have a better pharmacological profile compared to the first-generation ones, unfortunately these compounds show some disadvantages, indeed they may be responsible of unexpected drug-drug interactions and they are able to inhibit cytochrome P450 resulting in an increase of drug toxicity [74,91-93].

In order to improve the features of P-gp modulators/inhibitors, a third-generation of inhibitors with high affinity for ABC transporters, high specificity and potency has been developed. Third generation includes compounds such as tariquidar and elacridar [33]; tariquidar binds to P-gp through a non-competitive mechanism, while elacridar acts by binding to the allosteric site of P-gp [33,94].

Some studies show that the inhibition of P-gp improves brain drug delivery of some anticancer compounds and consequently the treatment of CNS tumors; Fellner et al. demonstrated that P-gp inhibition by valspodar increases paclitaxel brain levels in nude mice with intracerebrally implanted human U-118 MG glioblastoma [18,95]. Even if *in vitro* and *in vivo* studies report that these drugs are associated to an enhancement of chemosensitivity, unfortunately they do not appear associated with an improvement of OS in cancer patients [72,96,97], moreover they continue to show unexpected toxic effects [33,71].

Probably side effects and drug-drug interactions may be responsible of ABC transporter inhibitors failure, limiting their clinical application and their translation from animal model to patient. In addition, inhibiting a specific transporter, the remaining ABC transporters, that are coexpressed in the tumor, could compensate by their biological activity, interfering with inhibition and reducing the effectiveness of the drug inhibitor.

Some research studies tried to develop novel P-gp inhibitors which constitute the fourth generation [33,71]; substances belonging to this group are natural agents and their derivatives, surfactants and lipids, peptidomimetics. Surfactants are responsible of alteration of membrane lipids integrity and are able to modify P-gp structure [33,98] determining loss of P-gp function [33,98]; other substances act through other mechanisms such as limitation of P-gp ATPase activity [33,99]. Finally other agents associate transporters inhibition with another favorable biological function (dual ligands), for example some aminated thioxanthones were able to inhibit cell growth and P-gp activity at the same time [33,100].

Despite all the efforts to develop P-gp inhibitors, only few compounds have been evaluated for their capacity to enhance drug delivery into the CNS; currently researchers are trying to identify new approaches and to find new P-gp inhibitors or novel mechanisms of action, among which natural compounds, small molecule inhibitors, RNA interference and epigenetic regulation [72].

For instance, several TKIs are ABC transporters modulators and have been examined to allow drugs to overcome the BBB increasing their bioavailability; probably many TKIs are very functional for their capacity to inhibit both P-gp and BCRP at the same time. Intriguingly, gefitinib, a TKI, increased topotecan penetration into the brain ECF likely via inhibition of BCRP and P-gp [101-105]. Some *in vivo* or *in vitro* studies described other TKIs, such as nilotinib and icotinib, which appeared able to inhibit ABC transporters enhancing chemotherapeutic drugs efficacy [72,106-108].

Flavonoids, alkaloids, coumarins and terpenoids are natural compounds that *in vitro* or *in vivo* studies seem to be associated with P-gp downregulation and decrease of proteins expression; their application is promoted by low cost, low toxicity and action extended to other ABC transporters too [72,109-114]. Among these compounds curcumin, active principle of *Curcuma longa*, was examined in some *in vitro* models of breast, colon and prostate cancer and appeared to be associated to an increase of sensitivity to some drugs [72,115-118].

Another very interesting approach to fight multidrug resistance is given by the possibility to block and silence ABC transporters expression through siRNA or RNAi mechanisms, approaches which involve RNA molecules to inhibit gene expression or translation. RNAi technology was used for the first time in 2003 in human cancer cells to knockdown the P-gp encoding mRNA reversing chemoresistance [72]. Since then several *in vitro* studies, especially on gastric, pancreatic, lung and ovarian cancer, designed stable vectors to overcome chemoresistance decreasing P-gp and other ABC transporter expression, sensitizing cells to antineoplastic compounds and promoting drug accumulation [72,119-120]. LncRNAs is a novel class of transcripts which includes important regulators of transcriptional processes. The lncRNAs have a wide range of functions in cellular and developmental processes and are able to regulate several genes including genes associated to anticancer resistance.

Another strategy to fight MDR is represented by the use of microRNAs, which are endogenous, nonprotein-coding, short RNAs of 20–22 nucleotides, involved in gene expression regulation by the ability to bind mRNA and silence genes. The genes inhibited by microRNAs are involved in different biological processes such as embryogenesis, cell development, proliferation and apoptosis [121,122]. Interestingly, microRNAs are dysregulated in cancer. Researchers are investigating about the possibility of using these molecules as diagnostic or predictive or prognostic biomarkers in different tumors. Their alterations are involved at various levels of chemoresistance mechanism. Some studies identified a pattern of microRNAs responsible of the inhibition of P-gp expression in MCF-7 breast cancer cells and esophageal squamous carcinoma cells [74,123-125]. Other microRNAs are involved in ABC transporters inhibition improving sensitivity to antineoplastic agents, for example miR-122 in hepatocellular carcinoma cells [72,126], miR-19a and miR-19b in gastric cancer cells [72,127], miR-145 in ovarian cancer cells [72,128], miR-137 in neuroblastoma cells in which for instance it regulates the response to doxorubicin treatment [72,129]; intriguingly, miR-21 represents another molecule involved in the response, in particular it appears to promote doxorubic in resistance in GBM T98G cells [130].

Recently some studies identified a role of epigenetic modifications, such as DNA methylation and histone modifications, in the regulation of gene expression associated to chemosensitivity and resistance [72,131].

Finally, nanotechnology-based approach is being developed to overcome multidrug resistance, this approach consisting in constructs with the function to deliver chemical compounds, including drugs, such P-gp inhibitors, or miRNA or RNAi, to specific target cells. These constructs include liposomes, polymer and peptide/protein conjugates, polymeric micelles, polymeric, lipid and inorganic NPs; they show differences in structure, for instance liposomes are small artificial vesicles consisting of lipid bilayers, while NPs are carriers with natural or synthetic polimeric matrix. Concerning these constructs in general, even if they have different conformation, they all function as vehicle of chemical compounds without giving problems associated to high dosage, cell toxicity, low specificity and uptake. Delivery of antitumor molecules to cancer cells through a nanotechnology-based approach could be an interesting novel opportunity to inhibit P-gp expression enhancing intracellular drug concentration [72,74].

P-gp Contributes to Chemoresistance in Glioblastoma

Resistance to antineoplastic agents is the main reason for treatment failure of brain tumors, in particular resistance to TMZ is often quickly acquired by GBM cells; for this reason different studies try to understand molecular mechanisms underlying TMZ resistance with the aim of developing strategies to sensitize GBM cells to TMZ.

The first predictive and prognostic molecular biomarker linked to TMZ resistance is the enzyme MGMT implicated in DNA damage repair associated to TMZ action. According to Hegi et al., hypermethylation of MGMT promoter gene, and consequently its silencing, is observed in about 50% of GBM cases and is associated to a better prognosis and a longer survival regardless of the treatment [132]. Moreover, a major survival benefit was observed in patients with methylatated MGMT promoter treated with a combination of TMZ and radiotherapy compared to patients treated with radiotherapy only, suggesting an association between MGMT methylation and response to TMZ treatment in adult GBM patients.

Unfortunately, chemoresistance is a very complex mechanism linked to different biological processes; therefore MGMT promoter methylation status is not the only marker in GBM resistance. Sardi et al. studying methylation status of MGMT promoter and analyzing the expression of MGMT in pediatric brain tumors treated with TMZ, showed that MGMT was nearly always unmethylated in contrast to what is observed in adult brain tumors [133]; moreover the expression level of MGMT appeared variable. The unmethylated status of MGMT along with the involvement of other DNA repair mechanisms could justify the reduced efficacy of TMZ in pediatric brain tumors.

Another factor associated to chemoresistance in CNS tumors is the expression and the activity of efflux pump proteins, among these P-gp is the first identified xenobiotic drugs ATP-dependent efflux pump and the most examined.

Some studies investigated about the role of P-gp in TMZ resistance. Schaich et al. in GBM patients treated with TMZ, in order to investigate the possible involvement of MDR1 gene variants in patient's survival, discovered that the exon 12 C1236T polymorphism is predictive of the outcome independently from MGMT status [134]. This observation suggests that this polymorphism could play a role in patient response to TMZ; according to some hypotheses this effect could be associated to the genetic mechanism of linkage disequilibrium or to an altered affinity of P-gp for TMZ. In addition, *in vitro* analysis showed an increase of cytotoxicity and cell death in MDR1 negative cells after exposure to TMZ compared to MDR1-expressing cells. In the same study, as confirmation of the involvement of MDR1 in the resistance to TMZ, the authors observed a trend to restoration of chemosensitivity to the drug in MDR1-expressing cells when treated with a combination of TMZ and MDR1-inhibitor/modulator, especially cyclosporine A. Finally Schaich et al., reported a significant P-gp expression not only in parenchymal tissue but also in GBM vessels suggesting that drug delivery to the brain and drug resistance are influenced by the activity of P-gp of endothelial cells too [134].

In another study, using *in vivo* and *in vitro* models, authors tried to investigate the mechanisms underlying P-gp-mediated resistance to TMZ in GBM [135]. The authors distinguished an active and an inactive form of P-gp, both expressed in GBM cells; TMZ induced the active form of P-gp, indeed an increase of active P-gp was observed when GBM cells were treated with TMZ even if for a short time, the increase was greater for chronical treatment of GBM cells with TMZ. In the study was observed that the increase of active P-gp was induced by TMZ through a bifasic mechanism accomplished by two steps. Firstly, TMZ treatment promoted the traffic of active intracellular P-gp to the cell membrane directly; in a second phase the increase of P-gp was associated to an increase of transcription of MDR1 gene, induced by TMZ-mediated production of EGF, and subsequently to an increase of P-gp protein synthesis.

Regarding P-gp cellular traffiking, TMZ treatment reduced subcellular P-gp level and at the same time increased the active P-gp in the cell membrane, the activation of P-gp requiring a conformational change; moreover the increase of P-gp protein expression appeared to be time-dependent.

As far as the transcription of MDR1 gene is concerned, TMZ promoted this molecular mechanism increasing firstly production and release of EGF, thus promoting enhancement of EGFR signaling, resulting in the activation of the heterodimeric transcription factor AP-1 which in the end promotes the transcription of MDR1 gene. An increase of transcription MDR1 gene protected GBM cells from TMZ treatment with consequent increase of cell survival; as

confirmation of these results MDR1 knockdown cells showed an increase of cytotoxicity with a decrease of survival when treated with TMZ. Therefore through an autocrine mechanism TMZ-resistant GBM cells expressed EGFR and in the presence of TMZ produced EGF at the same time, in this way EGF stimulated the same cells inducing MDR1 gene expression through AP-1 activation.

In the same study, the authors observed also that on the other hand the use of kinase inhibitors, such as erlotinib, to block EGFR signaling in combination with TMZ reduced P-gp expression and promoted the action of TMZ on GBM cells, confirming the role and the involvement of EGFR signaling in the induction of MDR1 expression [135].Therefore concerning *in vivo* models they reported that combined therapy of erlotinib with TMZ promoted a decrease of tumor volumes, confirming the results obtained *in vitro*.

To summarize, TMZ activates cell surface P-gp, promotes protein expression and the enhancement of the function; combined therapy of TMZ with EGFR inhibitors prevents P-gp activation sensitizing GBM cells to TMZ treatment **(Figure 1).**

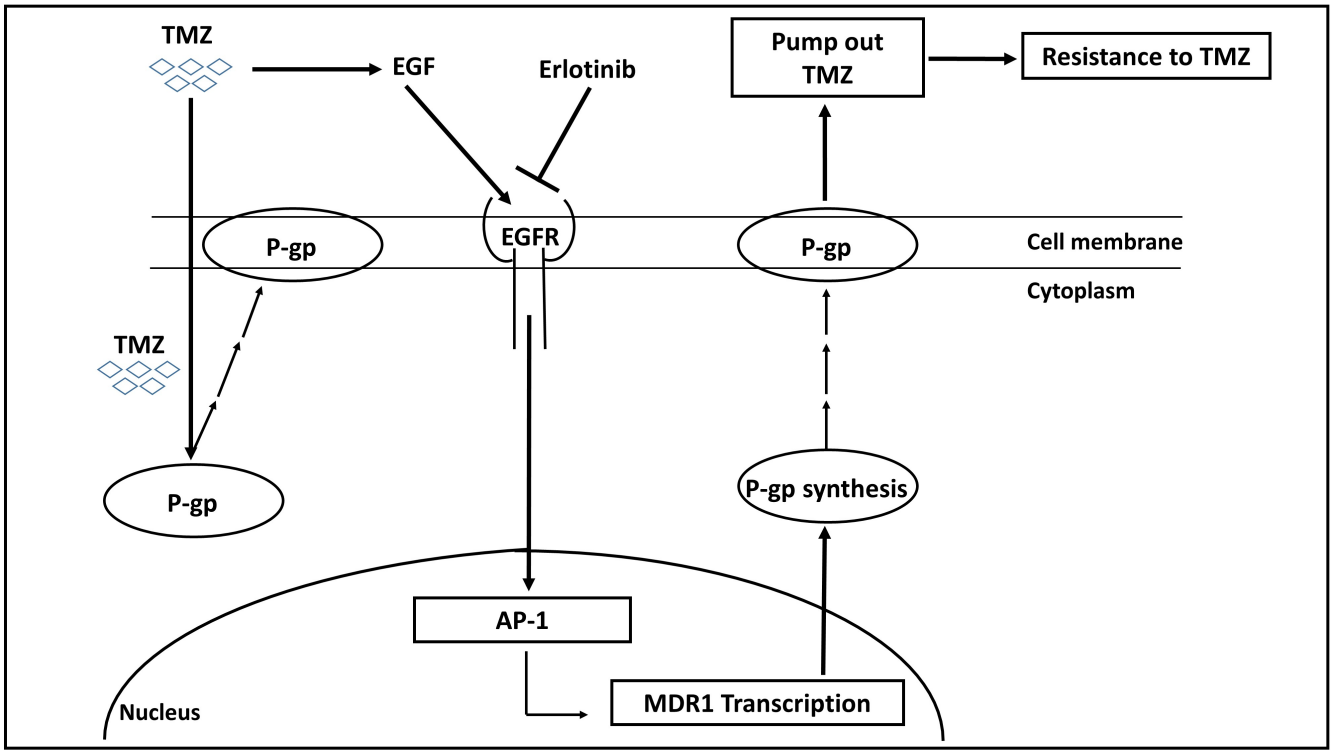

Figure 1. A diagram showing TMZ mediated P-gp synthesis, translocation and activity in TMZ-treated GBM cells. P-gp cellular trafficking, EGFR signaling activation, AP-1 activation, MDR1 transcription and erlotinib, an inhibitor of EGFR signaling, are represented.

In another report Munoz et al., using *in vitro* model, investigated about the interaction between P-gp and TMZ, in particular they co-administered P-gp fluorescent target with TMZ observing a competitive mechanism between TMZ and the other P-gp substrates [5]. According to the study, competitive mechanism was useful for combined treatment of GBM cells especially of TMZ with P-gp inhibitors, this association resulted in an increase of Caspase 3 activity reducing cell survival. Finally, using a computer modeling system, authors identified a specific region of interaction between TMZ and P-gp localized in the same area of interaction of other P-gp targets, and especially near ATP binding site.

Another study reported the involvement of miRNA-9 in TMZ resistance in GBM cancer stem cells CD133+ (prominin-1), this miRNA appeared connected to MDR1 gene [5,136]. In particular the authors observed that TMZ chemoresistance was associated to an increased level of miRNA-9 which promoted upregulation of MDR1 expression through activation of the SHH/PTCH1/MDR1 axis. Experiments carried out by targeted siRNA confirmed the involvement of SHH pathway in MDR1 upregulation. In general in brain tumors, some microRNAs appear involved in drug sensitivity/resistance. Giunti et al., using *in vitro* models, showed that miR-21, an oncogenic miRNA overexpressed in human breast cancer, was associated with resistance to doxorubicin in GBM T98G cells [130]; the authors observed a greater sensitivity of GBM T98G cells to doxorubicin, with an increase of apoptosis, when they were transfected with anti-miR-21 inhibitor compared to not trasfected control cells.

However, Zhang et al. showed in glioma cells and especially in P-gp overexpressed cells, an increase of sensitivity to P-gp substrates under the action of TMZ [137]. They reported that when TMZ was co-administered with doxorubicin, TMZ affecting P-gp activity promoted an increase of doxorubicin accumulation with a synergistic mechanism, suggesting that TMZ could reverse doxorubicin resistance improving the treatment efficacy. Doxorubicin is able to promote P-gp expression and in general the expression of the ATP-binding superfamily transporter proteins. *In vitro* models therefore confirmed synergistic effect between doxorubicin and TMZ showing an increase of doxorubicin accumulation in presence of TMZ, accumulation was significantly higher in presence of high dosage of TMZ. Analyzing the effect of TMZ on drug efflux pump, the authors showed that TMZ does not change P-gp protein expression, but acts inhibiting P-gp directly and especially decreasing P-gp ATPase activity. This mechanism of action could explain synergistic effect of combination between TMZ and doxorubicin; the mechanisms which promote accumulation of doxorubicin may represent a promising novel strategy against malignant gliomas (**Figure 2**) [137].

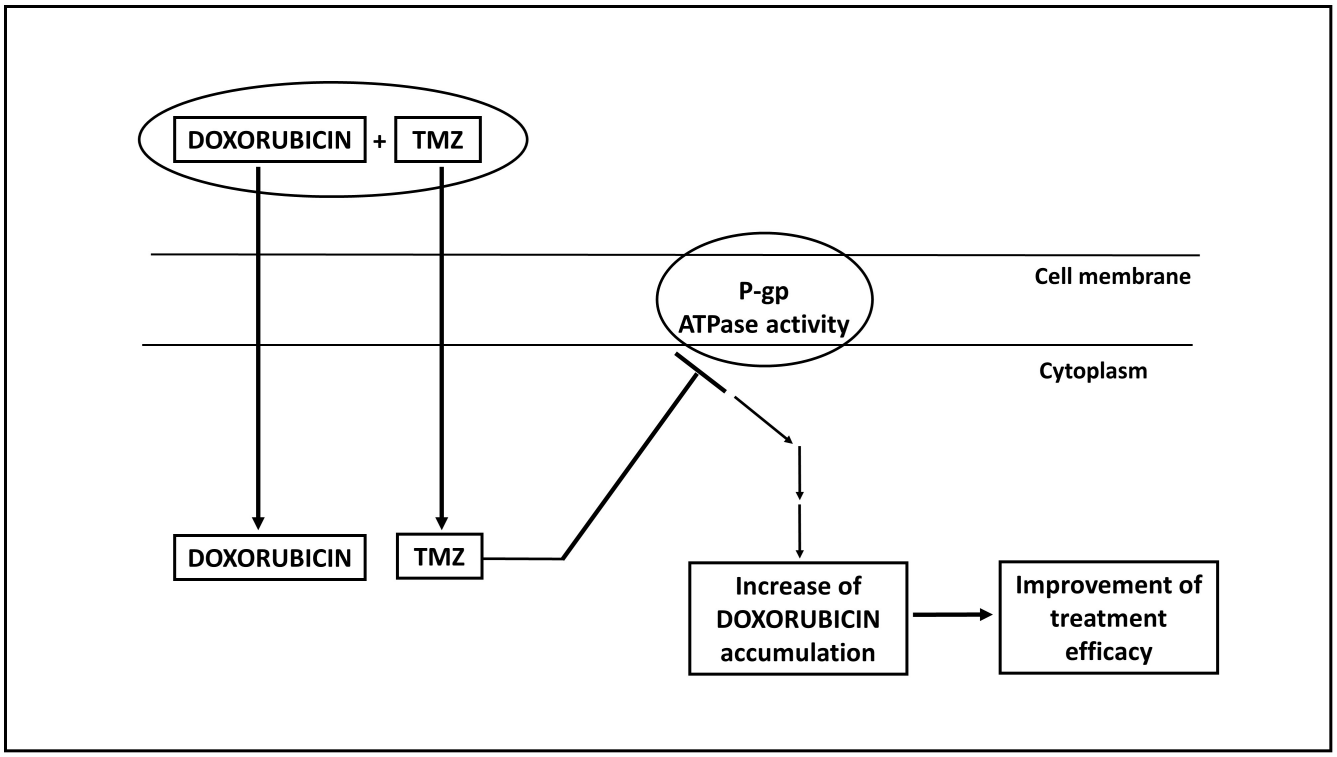

Figure 2. A diagram showing how TMZ, administered in combination with doxorubicin, promotes doxorubicin accumulation inhibiting P-gp ATPase activity and resulting in an improvement of treatment efficacy.

TMZ affecting P-gp sensitizes GBM cells to different drugs that are all substrates of P-glycoprotein; therefore in general drugs that synergized with TMZ are substrates of P-gp.

According to Riganti et al., also in GBM cancer stem cells TMZ promoted the accumulation of other drugs affecting P-gp activity, in particular TMZ appeared to methylate Wnt3a gene promoter reducing its expression [138]; Wnt3a is an important factor involved in cell growth, tumorigenesis and stemness maintenance. The diminished expression of Wnt3a was associated with a decrease of transcriptional activation of ABCB1 resulting in reduced P-gp protein expression and efflux pump activity. Therefore, through this mechanism, TMZ may sensitize GBM cancer stem cells to P-gp substrate promoting their accumulation in tumor cells and consequently their cytotoxic and antiproliferative effects.

Several P-gp inhibitors are not successful in clinical trials because they show low specificity and high toxicity, for all these reasons TMZ may represent an alternative strategy able to act as chemotherapeutic drug and chemosensitizer agent at the same time [138].

Concluding Remarks and Future Perspective

GBM is the most common and malignant primary brain tumor in adults with a dismal prognosis, a survival of up to 12-18 months and a very low possibility to survive longer than 5 years [137,139].

The main cause of the frequent relapse of this disease is due to chemoresistance of GBM stem cell. Generally GBM stem cell resistance is associated to alterations in different biological processes such as cell cycle, apoptosis, DNA repair; moreover, in brain tumors the BBB is the main

responsible of the difficulty for some chemotherapy agent to reach CNS, promoting drug resistance.

Chemoresistance is mainly associated to the activity of efflux pumps; in particular P-gp, BCRP and MRPs proteins work together on the BBB and on the plasma membrane of brain tumors cooperating and playing a relevant role in the MDR phenomenon.

TMZ represents the frontline treatment for GBM, so it is very important to understand the mechanisms underlying TMZ-resistance and find novel approaches to overcome it.

Recently some studies analyzed the key role of P-gp in drug resistance mechanism observed in GBM and especially its implication in TMZ resistance. These reports showed the involvement of genetic variant of MDR1 gene in response to TMZ and the implication of TMZ in P-gp activation; in particular TMZ seems to promote P-gp expression and function. However, targeting P-gp increases sensitivity to TMZ resulting in an increase of apoptosis and therefore reversing the resistance to TMZ. In addition TMZ competes with P-gp substrates, including some antineoplastic agents, representing a promising opportunity for combined targeted therapies.

Further preclinical and clinical investigations are necessary to better understand and overcome resistance mechanism of GBM to TMZ associated to P-gp and other MDR mechanisms, in order to develop novel therapeutic strategies and new molecules or optimize combined therapies for the treatment of CNS tumors.

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